

## ORIGINAL PAPER

## An amplified sandwich EIA for the measurement of soy aeroallergens

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Clinical and  
Experimental  
Allergy

## Summary

**Background** Soy hull low-molecular-weight (SHLMW) allergens were responsible for the soy asthma epidemics in Barcelona, with one 7.5 kDa protein (Gly m 1) being the main IgE-binding component. The aims of this study were to develop a sensitive sandwich enzyme immunoassay (EIA) using rabbit polyclonal antibodies to measure low levels of SHLMW allergens, and to compare this method with the previously described human IgE EIA-inhibition technique.

**Methods** IgG was isolated from serum of rabbits immunized with a chromatographically purified SHLMW extract (SHLMWE). Antibody-binding profiles were compared with those of human IgE anti-soy protein antibodies by Western blot analysis. An amplified sandwich EIA was developed using the purified SHLMWE as a calibration standard. Results were expressed in nanograms per millilitre. To compare the two assays, 54 air samples were analysed by both methods.

**Results** SDS-PAGE of the SHLMWE revealed four bands of 6, 8, 15 and 17 kDa. Gly m 1 in the SHLMWE was identified by fingerprinting. The detection limit of the assay was 40 pg/mL. The two methods correlated well ( $r = 0.89$ ;  $P < 0.001$ ). The allergen concentration was detected in all 54 (100%) samples by the sandwich EIA but in only 37 (68.5%) by the EIA inhibition.

**Conclusions** The amplified sandwich EIA for SHLMW components has a high sensitivity and appeared to be a useful tool for the measurement of airborne SHLMW allergens, even at relatively low concentrations. Moreover, the method uses rabbit antibodies at high dilutions and does not require human sera, with limited availability and quantitative and qualitative pool-to-pool variability.

**Keywords** airborne, polyclonal antibody, soy hull

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## Introduction

Epidemic asthma due to soy dust emissions has been described [1–3]. Investigation into these epidemics revealed that the outbreaks were caused by soy dust generated during soy unloading in the harbour [1,4]. Occupational asthma related to soy proteins has also been described [5–8], indicating that asthma, rhinitis and other allergic manifestations can be caused by inhalation of soy derivatives in predisposed individuals.

The allergen responsible for the soy asthma epidemics was partially characterized by Rodrigo et al. [9], who found that the serum of patients affected by soy epidemic asthma reacted specifically to a low-molecular-mass pro-

tein ( $< 14$  kDa) mainly present in the soy hulls. Later, two soy hull proteins were shown to be the main allergens responsible for these asthma epidemics [10]. These proteins are isoallergens (Gly m 1A and Gly m 1B) with molecular weights (MW) of 7.0 and 7.5 kDa, respectively, and are highly homologous with the hydrophobic soy protein (HSP) described by Odani et al. [11]. Although other proteins such as trypsin inhibitor (20 kDa) have been described as important inhalant soy allergens in occupational asthma [12], only four proteins are currently included in protein databases as major soy allergens [13].

The dose-response relationships implicated in sensitizing a person and eliciting symptoms in sensitized people are unknown; nevertheless, several studies have shown

the importance of reducing soy emissions to prevent asthma attacks [4, 14, 15]. To this end, it would be useful to have highly sensitive methods for routine monitoring of airborne soy allergens levels, which can be quite low outdoors and at the workplace [15, 16].

Several immunoassays have been described for quantifying airborne soy allergens. Two of these are inhibition assays that use pooled human serum [16, 17]. Nevertheless, these assays have a relatively low sensitivity and potential long-term reproducibility problems. A monoclonal antibody-based method to quantify Gly m 1 with a higher sensitivity than inhibition methods has also been reported [18]. One disadvantage of monoclonal antibody systems is that each monoclonal only recognizes a single epitope of the allergen and, in addition, the technology to raise monoclonal antibodies is more costly than producing polyclonal antibodies [19]. There are positive advantages of using polyclonals, e.g. cheaper and easier, the polyclonal method is less disturbed by epitope losses, several allergens may be present that can be detected and polyclonal methods can be made very sensitive.

In an effort to improve the quantification of soy allergens, this study was designed to develop a sensitive sandwich enzyme immunoassay (EIA) using rabbit polyclonal antibodies for the measurement of low levels of soy hull low-molecular-weight (SHLMWE) allergens and to compare this method with the previously described human IgE EIA-inhibition method currently used to measure soy hull aeroallergen in the city of Barcelona [16].

## Materials and methods

### *Preparation of soy hull extracts*

**Soy hull low molecular weight extract.** The SHLMWE extract (SHLMWE) was obtained from soy hull with a chromatographic process (CM-cellulose and DEAE-cellulose chromatography) based on the protocol described by Odani et al. [11] to obtain HSP. Large quantities of SHLMWE were obtained before starting the study in order to ensure continuity of this reagent. The protein concentration in the extract was 1 mg/mL, as determined by the bicinchoninic acid (BCA) method, using bovine serum albumin (Pierce Chemical Co., Rockford, IL, USA) as the reference.

**Complete soy hull extract.** Briefly, in order to obtain the complete soy hull extract (CSHE), soybean hull proteins were extracted in 0.1 M  $\text{NH}_4\text{HCO}_3$  buffer. The eluate was filtered, dialyzed in a 3.5 kDa cut-off membrane and lyophilized. The protein concentration in the extract was 34.4% as determined by the BCA method. The SHLMWE content in CSHE was 6.6% referred to the total of proteins.

### *Characterization of soy hull extracts*

**Electrophoresis.** One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the SHLMWE and the CSHE proteins was performed in a Novex Xcell II MiniCell electrophoresis unit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Proteins (20 µg/lane) were dissolved in sample buffer and incubated for 10 min at 70 °C before loading on a precast 10% NuPAGE gel (Novex, Invitrogen). Electrophoresis was performed under reducing conditions at 200 V for 30 min. SeeBlue™ Plus2 pre-stained standards, ranging between 3 and 188 kDa (Invitrogen), were used as markers. Proteins were stained with Coomassie brilliant blue (Pierce Chemical Co.), and the MW of the protein bands was calculated by an image analysis system (Lane Manager v 2.1, TDI, Madrid, Spain).

**Protein identification.** Protein identification was performed by peptide mass fingerprinting. Briefly, SHLMWE and CSHE protein bands separated by one-dimensional SDS-PAGE were excised from the gel and digested with trypsin using an automatic digester (Investigator™ Pro-Gest Protein Digestion Station – Genomic Solutions, Ann Arbor, MI, USA). The peptide mass fingerprint was produced by analysing the digested protein with a mass spectrometer MALDI-TOF/TOF (Proteomic Analyzer; Applied Biosystems, Courteboeuf, France) or capLC-Q-TOF (Waters/Micromass; Manchester, UK); data were obtained with the MASCOT search engine.

### *Production of anti-SHLMWE polyclonal antibodies and biotinylation*

Immunoconjugates were prepared by coupling 5 mg of SHLMWE to the carrier protein, keyhole limpet hemocyanin (KLH) (Pierce Chemical Co.), following the instructions of the manufacturer. The coupled products were purified by fast-protein liquid chromatography (FPLC). Two male New Zealand white rabbits were injected subcutaneously with 1 mL of SHLMWE-KLH (100 µg) mixed with an equal volume of Freund's complete adjuvant. Three booster injections with the same allergen concentration in Freund's incomplete adjuvant were administered at 4-week intervals. Use of the animals for this purpose was approved by the Animal Ethics Committee of the center.

The IgG fraction of the polyclonal antiserum was isolated on an immobilized Protein A-agarose column (Pierce Chemical Co.), and then applied to an Excellulose column (Pierce Chemical Co.) to desalt it and exchange the buffer to PBS. The protein concentration of the IgG fraction was 3.3 mg/mL, as determined by the BCA method (Pierce Chemical Co.). The antibody titre estimated by EIA was about 1:40 000. The antibody was stored at –20 °C.

A portion of polyclonal antibody against-SHLMWE was biotinylated using the EZ link<sup>®</sup> Sulfo-NHS-LC-Biotinylation Kit (Pierce Chemical Co.) according to the manufacturer's instructions. Biotin incorporation was quantified by measuring the absorbance of the HABA-avidin solution (Pierce Chemical Co.) before and after addition of the biotin. The biotin ratio was 7.32 moles of biotin/mole of protein.

### Immunoblotting

For immunoblotting, SHLMWE and CSHE were separated by SDS-PAGE, as described above. The MW markers were excised and the remaining gel was blotted onto a nitrocellulose membrane (0.22 µm pore size, Bio-Rad, Richmond, CA, USA) and washed once with PBS (0.1 M phosphate buffer, pH 7.4). After blocking the unoccupied binding sites with 5% blotting grade blocker non-fat dry milk (Bio-Rad) for 1 h under gentle agitation, the membrane was washed three times with PBST (PBS with 0.1% Tween 20). The membrane was incubated with 1 : 10 000 polyclonal anti-SHLMWE antibody or a 1 : 10 pool of sera from allergenic patients with 19 kU/L specific IgE to soybean (human IgE) diluted in PBST for 1.5 or 2 h, respectively, at room temperature under gentle agitation. After three washes with PBST, the membranes previously incubated with the polyclonal anti-SHLMWE antibody were incubated for 1 h with 1 : 20 000 diluted goat-anti-rabbit IgG (H+L) labelled with HRP (Southern Biotechnology Associates, Inc., Birmingham AL, USA). The membrane previously incubated with human IgE was incubated for 1 h with 1 : 10 000 diluted biotinylated mouse-anti-human IgE (0.5 mg/mL, clone B3102E8; Southern Biotechnology Associates); after three washes, the membrane was incubated for 1 h with 1 : 10 000 diluted streptavidin-HRP (Southern Biotechnology Associates) at room temperature. After further washing, all the immunoblots were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.).

### Anti-SHLMWE amplified sandwich EIA protocol

Wells of high-binding microtitre plates (Costar, Cambridge, MA, USA) were coated with the polyclonal antibody against SHLMWE (0.1 µg protein/well) in PBS buffer, pH 7.4, overnight at 4 °C. The wells were blocked (200 µL/well) with PolyHRP diluent (Research Diagnostics, Inc., Flanders, NJ, USA) for 1 h at 37 °C. After washing three times with PBST (PBS with 0.1% Tween 20), plates were incubated for 1 h at 37 °C with 100 µL/well of either filter elutes, SHLMWE standards containing 0.04–5 ng/mL and controls, diluted in Poly-HRP diluent. After three washes with PBST, plates were incubated for 1 h at 37 °C with 100 µL/well of biotinylated polyclonal antibody against-SHLMWE at 1 : 2000 dilution (0.45 µg/mL) in Poly-HRP diluent and washed again three times with PBST. Poly-

HRP80-Streptavidin (Research Diagnostics Inc., Flanders, NJ, USA) (1/15 000) in Poly-HRP dilution buffer was added. After 45 min at room temperature under gentle agitation, wells were washed three times and the reaction was developed with 0.3% H<sub>2</sub>O<sub>2</sub>, 3,3',5,5'-tetramethylbenzidine in acetate buffer (pH 5.5) at room temperature in the dark. After 20 min, the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and read at 450 nm with a plate reader (Titertek Multiskan Plus MKII). The standard curve was constructed from eight data points using a four-parameter logistic curve fit. Results are expressed in nanograms per milliliters, referring to the protein content of the standard preparation. Genesis software (version 2.16, Life Sciences Ltd, Basingstoke, UK) was used for calculating the results.

The intra-assay coefficient of variation (CV) was calculated by using two controls at a low and a high concentration of SHLMWE (0.15 and 0.62 ng/mL, respectively). Thirty replicates of each control were analysed in one assay. The inter-assay CV was assessed by analysing the two controls in duplicate on 10 different days during a period of one month, in which all assays reported in this paper were performed. Thus, the CV (inter) for each was defined as the relative standard deviation ( $100 \times \text{SD}/\text{mean}$ ) of the means of 10 duplicate measurements.

The lower limit of detection was defined as the concentration of the lowest point on the standard curve, with the optical density (OD) value at this point having to be 0.05 above the blank. As the corresponding OD at this concentration (40 pg/mL) was on average 0.108, and 10 SDs above the OD of the blank, the risk of false-positive test results due to measurement errors was very low. The lower limit of quantification was defined as the concentration, which, according to the curve fitting function, gave the OD of the lowest point on the standard curve plus 10% of the highest point on the standard curve. The upper limit of quantification was defined as the concentration, which, according to the curve fitting function, gave 90% of the OD at the highest point on the standard curve. Under these premises, the determinations are largely limited to the log-linear part of the curve.

### Specificity of the assay

As soy belongs to the legume family, the cross-reaction study to assess the specificity of the assay was performed using extracts from several common legumes (green bean, green pea, chickpea and peanut), other legumes and cereals unloaded in the harbour, such as corn, rye, field pea and wheat fodder, and the following outdoor allergens: *Aspergillus fumigatus*, *Penicillium notatum*, *Platanus hybrida* and *Parietaria judaica*. The legume and cereal extracts were obtained by soluble protein extraction with 0.1 M NH<sub>4</sub>HCO<sub>3</sub> buffer and dialysis against a 3.5 kDa pore size dialysis membrane (Spectrapor, Iberlabo, Los Angeles, CA, USA). Subsequently, the extracts were characterized

by SDS-PAGE. Pollen and mould extracts were commercially available (Laboratorios Leti, Madrid, Spain).

#### *Comparison between IgE EIA inhibition and anti-SHLMWE sandwich EIA*

Fifty-four samples were collected from the air of the city of Barcelona with a large-volume automated air sampler (CAV-A/HF, MCV, SA, Barcelona, Spain) containing glass microfibre filters with a 1 µm pore size (Whatman International Ltd., Maidstone, UK), installed close to the harbour and changed daily. Dust collection was performed at a flow rate of 55 m<sup>3</sup>/h. Soy aeroallergens were extracted from the filters in 5 mL PBS/0.2% BSA/0.1% Tween 20 (pH 7.4) overnight at 4 °C. Filters were discarded and the eluates were stored at -20 °C. The 54 eluates were analysed by the anti-SHLMWE sandwich EIA, using the SHLMWE as a standard, and a human IgE inhibition EIA previously described by Cruz et al. [16] as the reference method, using the Soy hull low molecular mass extract (SHLMWE) as a standard, and values were compared. Owing to the variability of these methods, specific criteria were established for accepting the results. Only values with a CV (%) in the same assay less than 20% in duplicates were considered acceptable.

#### *Statistical analysis*

The percentage of positive samples, and the median and range of soy aeroallergen levels were calculated. Samples with undetectable levels were assigned the value of the lower limit of detection. A one-sample Kolmogorov-S-

mirnov test to assess normality was used for soy aeroallergen-positive samples measured by both immunoassays. The Kolmogorov-Smirnov test showed a normal distribution; hence, the Pearson rank test was applied to determine the correlation between the two methods. Agreement between methods was determined by calculating the geometric mean of the ratio among soy aeroallergen levels detected by both methods in positive samples, and the 95% confidence interval (CI) for the mean ratio was calculated [20]. SPSS release 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

## Results

### *Characterization of SHLMWE and immunoblotting*

Figure 1 shows the SHLMWE and CSHE protein bands separated by SDS-PAGE. A total of four bands with MWs ranging from 5 to 14 kDa were observed for SHLMWE, and bands of 5 to 99 kDa were observed for the CSHE. MALDI-TOF/TOF or Q-TOF mass spectrometry identified three SHLMWE protein bands as HSP (Table 1), whereas band 1 (Fig. 1) was too faint to be analysed. For the CSHE, bands 8 and 9 were identified as HSP. Bands 2 and 3 of SHLMWE and band 8 of CSHE are polymeric forms of the hydrophobic seed protein. This is the reason why they have a different molecular mass even though they are the same protein. Western blotting showed that the polyclonal anti-SHLMWE antibody and the human IgE reacted only to the HSP of the extracts (Fig. 1).

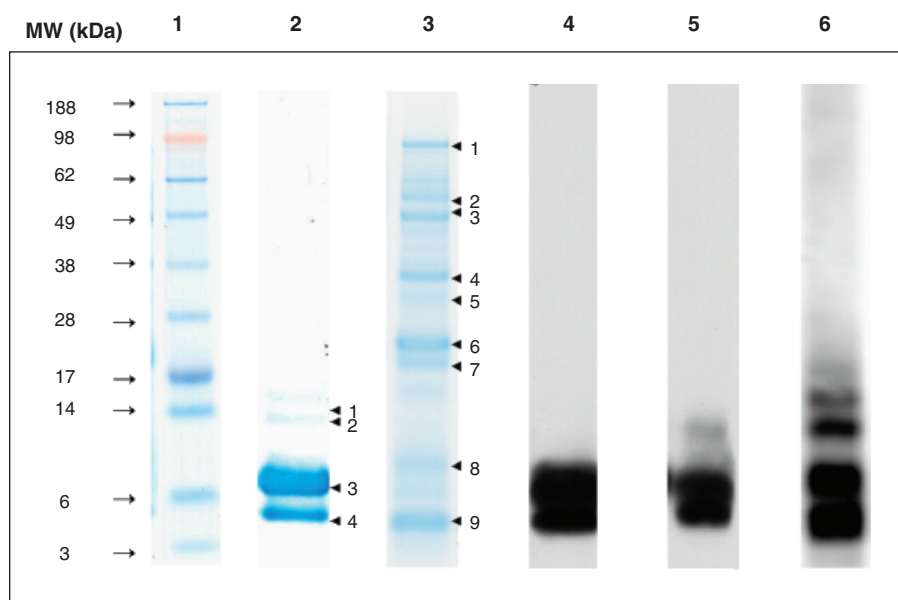


Fig. 1. Soy hull low-molecular-weight (SHLMWE) and complete soy hull extract (CSHE) electrophoresis and immunoblotting with the polyclonal anti-SHLMWE antibody and human IgE. Lane 1, molecular weight markers; lane 2, Coomassie-stained SDS-PAGE of SHLMWE; lane 3, Coomassie-stained SDS-PAGE of CSHE; lanes 4 and 5: SHLMWE and CSHE immunoblots with the polyclonal anti-SHLMWE antibody, respectively, and lane 6, SHLMWE immunoblot with human IgE.

**Table 1.** Identification of SHLMWE and CSHE proteins by MALDI-TOF-TOF or Q-TOF mass spectrometry

| Band id* | MW (KDa) <sup>†</sup> | Protein identified (theoretical MW in KDa) <sup>‡</sup> | Accession number (GI) <sup>§</sup> | Sequence coverage (%) <sup>*</sup> | Sequence <sup>  </sup>                                      |
|----------|-----------------------|---------------------------------------------------------|------------------------------------|------------------------------------|-------------------------------------------------------------|
| SHLMWE   |                       |                                                         |                                    |                                    |                                                             |
| 2        | 11.60                 | Hydrophobic seed protein (8.8)                          | 123506                             | 37                                 |                                                             |
| 3        | 6.62                  | Hydrophobic seed protein (8.8)                          | 123506                             | 37                                 |                                                             |
| 4        | 5.18                  | Hydrophobic seed protein (8.8)                          | 123506                             | 37                                 |                                                             |
| CSHE     |                       |                                                         |                                    |                                    |                                                             |
| 1        | 98.6                  | Lypoxigenase (97.1)                                     | 81793                              |                                    | SNDVYLPR/ELVEVGHGDK/YSVEMSAVVYK                             |
|          |                       | β-conglycinin α' subunit (45)                           | 28629838                           |                                    | SQQLQNLN/SRDPIYSNK                                          |
| 3        | 56.7                  | α subunit of β-conglycinin (63.1)                       | 9967357                            |                                    | GAIVTVK/LNALKPDNR/NLQGENEGEDK/<br>SQSDNFEYVSFK/EQPQQNECQIQK |
|          |                       | β-conglycinin β-subunit (50.5)                          | 15425635                           |                                    | SQQLQNLN/NILEASYDTK                                         |
| 4        | 38.7                  | Glycinin A1aBx (56.3)                                   | 9967357                            | 17                                 |                                                             |
|          |                       | α' subunit of β-conglycinin (65.2)                      |                                    |                                    |                                                             |
| 5        | 29.6                  | Precursor lectina (agglutinin) (30.9)                   | 126151                             | 24                                 |                                                             |
| 6        | 17.2                  | Glycinin A5A4B3 precursor (30.9)                        | 81785                              |                                    | ADFYNPK/QSQVSELK/THHNAVTSYLK/<br>ISTLNSLTLPALR              |
|          |                       | Kunitz trypsin inhibitor (24.3)                         | 13375349                           |                                    | GIGTISSPYR/NKPLVVQFQK/<br>FIAEGHPLSLK/VSDDEFNNYK            |
| 8        | 7.8                   | Hydrophobic seed protein (8.8)                          | 123506                             | 37                                 |                                                             |
| 9        | 5                     | Hydrophobic seed protein (8.8)                          | 123506                             | 37                                 |                                                             |

\*Each band is described by its number in the SDS-PAGE (Fig. 1).

<sup>†</sup>Apparent MW extrapolated from the calibration curve.

<sup>‡</sup>Proteins were identified by MALDI-TOF-TOF mass spectrometry.

<sup>§</sup>Accession number in the NCBI database.

<sup>\*</sup>Percentage of the protein covered by matched peptides.

<sup>||</sup> Sequence obtained by capLC-Q-TOF.

### Amplified sandwich EIA

The standard curve of the optimised assay with different concentrations of SHLMWE followed a typical sigmoidal shape. The anti-SHLMWE sandwich EIA detected a range of concentrations from 0.04 to 5 ng/mL (final concentrations in the well), but the working range defined by the lower and upper limit of quantification was between 0.10 and 1.82 ng/mL. The intra-assay coefficient of variation (CV) using the low and high controls was 4% and 5% and the inter-assay CV was 18% and 20%, respectively. The amplified sandwich EIA showed cross-reactivity, not only with soy grain, the origin of the SHLMWE, but also with field pea. Moreover, cross-reaction was also observed with some cereal extracts (corn, rye and wheat fodder), although only at very high protein concentrations. It was necessary to reach 50% of the OD of the highest point on the standard curve: 1500, 1900 and 1600 ng/mL, respectively. The other extracts tested did not react in the assay (Fig. 2).

### Comparison between IgE EIA inhibition and anti-SHLMWE sandwich EIA

Soy allergen concentrations above the lower LOD could be measured in 54 (100%) samples by the anti-SHLMWE

sandwich EIA but only 37 (68.5%) by IgE EIA inhibition. The median (range) (ng/mL) of the 54 samples was 9900 (2190–96 200) for IgE EIA inhibition and 129 (1.5–2350) for anti-SHLMWE sandwich EIA. The values measured by both assays correlated very well, with a Pearson's correlation coefficient for positive samples in both assays of 0.89 ( $P < 0.001$ ). The absolute values measured with the inhibition IgE EIA were, however, much higher, as shown by the relative difference plotted against geometric mean of soy aeroallergens levels found by the two immunoassays in the 37 positive samples (Fig. 3): the human IgE inhibition EIA yielded, on average, 68.5 times (95% CI 63.3–84.8;  $n = 37$ ) higher nominal values than the amplified sandwich EIA.

### Discussion

Respiratory allergies associated with exposure at work or in other environments to specific allergens are a common cause of asthma. A recent study demonstrated that approximately 1 in every 10 cases of asthma is work related [21]. Sometimes, the sensitizing allergens affect the general population and cause allergic asthma, as has occurred in the case of castor bean [22] and soy [1–3].

The severity of the soy asthma epidemics in Barcelona during the 1980s highlighted the need to adopt corrective

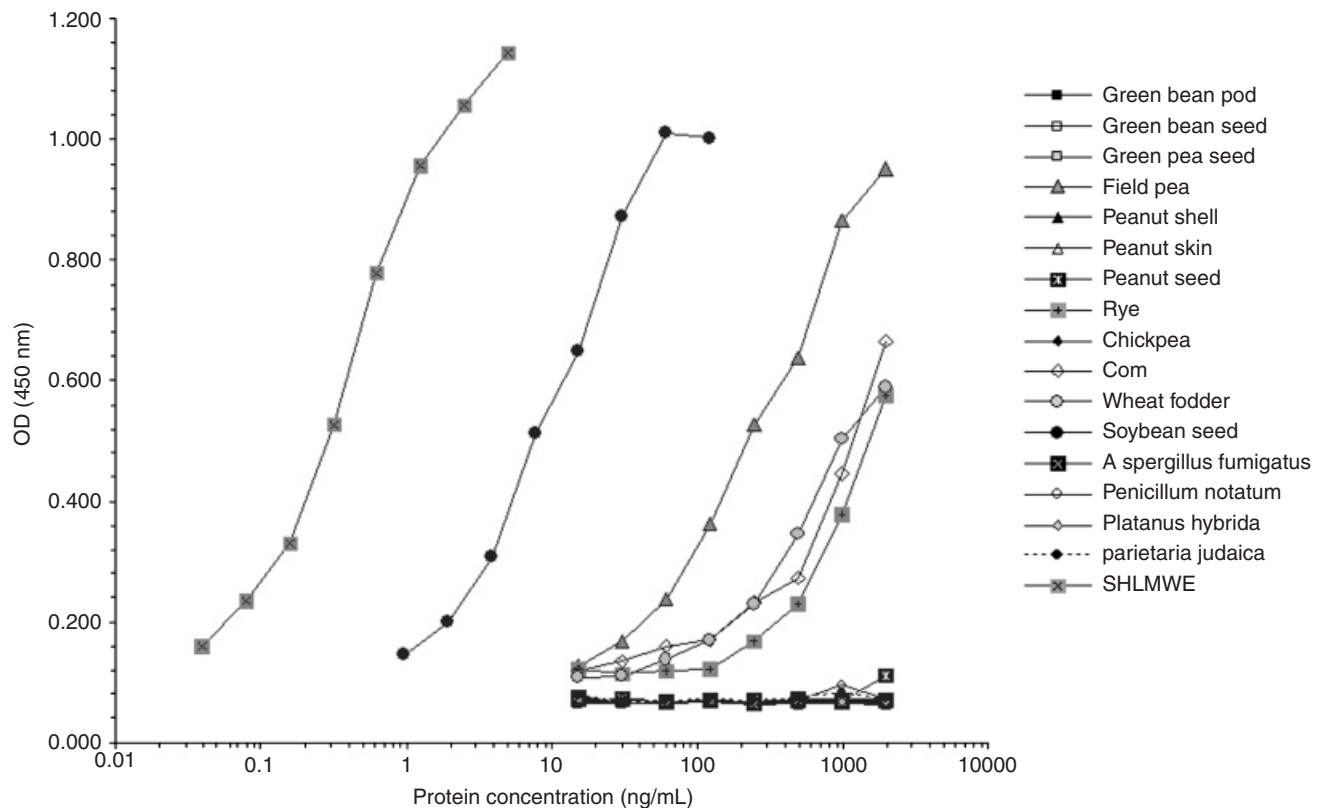


Fig. 2. Specificity of the assay tested using extracts of legumes, cereals, pollens and moulds. A parallel curve was obtained by using the soybean grain extract (x-axis on the log scale).

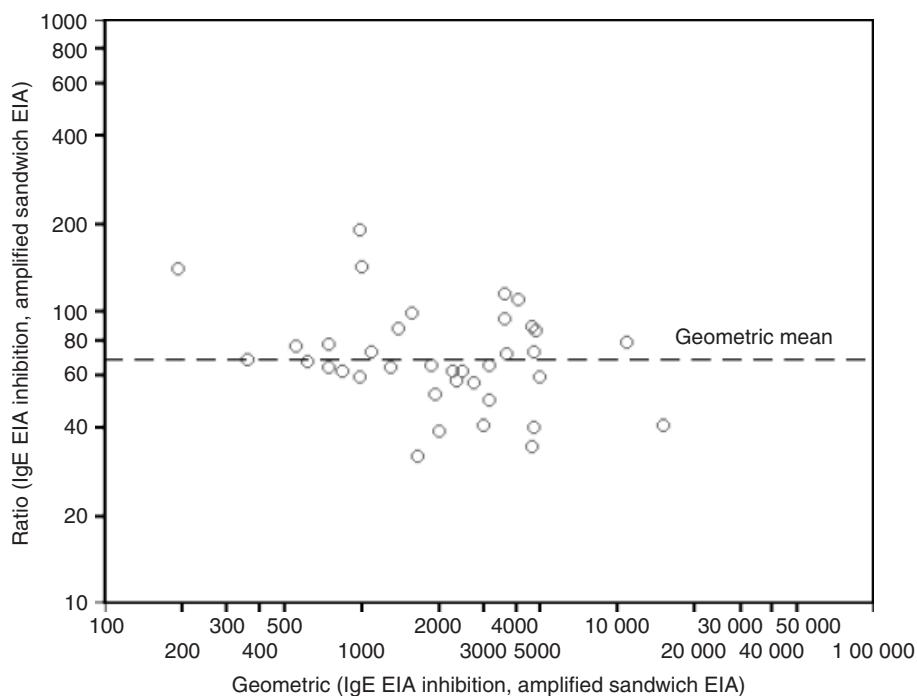


Fig. 3. Relative difference (ratio) plotted against the geometric mean of soy aeroallergen levels measured by both methods in 54 samples. The dashed line gives the geometric mean of ratios.

measures when unloading this legume. Despite the initial effectiveness of these measures specific IgE persisted in affected patients due to the presence of low concentrations of soy aeroallergens [4]. The development of a highly sensitive method for measuring airborne soy allergens would allow assessment of the efficacy of corrective measures applied to reduce the airborne levels of this allergen and investigation into the dose-response ratio in affected patients [23].

In this paper, we describe a highly sensitive amplified sandwich EIA (detection limit 0.04 ng/mL) based on polyclonal rabbit antibodies for the measurement of soy allergens. Besides conferring a capability to detect low concentrations of the allergen, the high sensitivity of this technique also permits a shorter sampling time, which is an advantage for experimental studies and makes investigation into exposure peaks possible.

Hitherto, three methods have been described for measuring environmental concentrations of soy allergens. Two of them are EIA-inhibition methods that use specific IgE antibodies from soy-sensitized humans [16, 17], and the third is a sandwich EIA method that uses mouse monoclonal antibodies to Gly m 1, but that, to our knowledge, is not generally available [18]. The use of pooled human serum in the inhibition methods is open to potential problems of long-term reproducibility due to heterogeneous mixtures [24]. This makes comparison of absolute aeroallergen concentrations among different studies difficult, as the efficacy of the technique has to be established for each antigen [25, 26]. Sandwich EIA is the most useful EIA technique for detecting soluble antigen, as it is fast, reproducible and two to five times more sensitive than competitive EIAs [27]. Nevertheless, the use of highly specific monoclonal antibodies should be examined with caution. As these antibodies only recognize one epitope of the protein, they may provide an underestimation of allergen levels by failing to detect other clinically relevant epitopes [19]. Because of this weak point, a technique using monoclonal antibodies should not necessarily be considered the method of choice. This problem might be circumvented by using mixtures of monoclonal antibodies (oligoclonal systems), which would avoid hyperspecificity and allow highly sensitive assays with an extended standard range, but the development of such systems would be very expensive. This approach has been described, for example, in the measurement of cytokines such as IL-6 or TNF- $\alpha$  [28]. Nevertheless, to our knowledge, there are no data in the literature regarding the application of oligoclonal systems to the study of allergens.

Several studies have recognized the need to attain a standardized method that gives a good representation of the allergen load [24]. As the main requirements for analysing airborne allergens are high enough sensitivity, intra- and inter-laboratory reproducibility and high specificity, sandwich EIA based on polyclonal antibodies

could become the method of choice. Polyclonal antibodies often have higher affinity than monoclonal antibodies, are much more economical to produce and can be as specific as monoclonals. Polyclonal antibodies are a mixture of antibodies that recognize different epitopes in the sample. How specific the polyclonal is depends on the antigenic extract used for rabbit immunization characteristics and how the antibodies are purified, as well as the antigenic nature of the allergens. In this study, the antibodies obtained were against Gly m 1 allergen, which is present in soy hull and considered to be the major allergen involved in the asthma epidemics due to this legume [11]. In the cross-reactivity study with other legumes and cereals, elevated specificity was demonstrated (Fig. 2). Field pea seems to lightly cross-react with soy, probably because field pea and soy have common storage proteins, which are characteristics of dry legumes. In accordance with this, we found a similar protein pattern in the SDS-PAGE between field pea and soy (data not shown). Furthermore, there was no cross-reaction between green pea and soy in our experiments. This type of assay can be quite sensitive, have a higher reproducibility than inhibition techniques and present an acceptable CV for this type of method [29, 30].

In the present study, there was a good correlation between the human IgE inhibition EIA and the anti-SHLMWE amplified sandwich EIA ( $r=0.89$ ). Nevertheless, the values obtained with the inhibition EIA were on average approximately 70 times higher than those found with the sandwich EIA, and these differences should always be accounted for when performing quantitative comparisons between samples assessed with different methods. Similar differences – even up to several orders of magnitude – were also observed by Swanson *et al.* [31] in a study measuring mite aeroallergens with various antibody detectors: monoclonal and polyclonal antibodies obtained in rabbit and human serum. Results with human serum were approximately 20-fold higher. Later, Renström *et al.* [24] performed a comparative study between two methods to assess rat allergens. The absolute allergen concentrations differed by several orders of magnitude between the methods, although there was a good correlation between them, as was also seen in our study. The magnitude of differences could also be attributed to the lack of pure reference standards that allow expression of the results in concentration of the antigen and the fact that many assays are validated in a limited number of settings; differing results might be obtained depending on the composition of the allergens present in the environment where the samples are collected [24, 29]. Because of these facts, validation and comparison of the assays and standardization of work conditions are essential to allow transferability of the results among laboratories.

In conclusion, the sandwich EIA described here has a high sensitivity and specificity and appears to be useful for measuring SHLMW aeroallergens, even at relatively low

concentrations. Moreover, as the method uses only rabbit antibodies at relatively high dilutions, it does not require human sera, which have limited availability and inevitable quantitative and qualitative pool-to-pool variability.

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